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Bacterial Endotoxin Isolated from a Water Spray Air Humidification System as a Putative Agent of Occupation-Related Lung Disease

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Outbreaks of hypersensitivity pneumonitis or humidifier fever were attributed to the inhalation of organic material aerosolized by a chilled-water spray humidification system. The purpose of this study was to isolate and characterize the serologically detectable antigen(s) present in extracts obtained from the humidification system. By using bicarbonate or phenol-water extractions or both, the antigen was isolated and characterized, using colorimetry, gas-liquid chromatography, reverse-phase high-performance liquid chromatography, and X-ray fluorescence. Carbohydrates, hexosamines, phosphorus, and even-numbered saturated and unsaturated fatty acids were constituents of the serologically detectable antigen. When tested in in vivo and in vitro assays, the antigen had demonstrable endotoxin activity. All subjects with biopsy-proven lung disease and a majority of subjects suspected of having lung disease had antibodies directed toward the purified endotoxin. The data strongly suggest that an aerosolized bacterial endotoxin is a putative agent inducing lung disease.

It has been reported that cool mist vaporizers, humidifiers, and water spray air conditioners are potential reservoirs of organic material capable of inducing lung disease (3, 11, 14, 15, 17, 25). The etiological agents of disease have been reported to be thermophillic actinomycetes (31), amoebae (16), and endotoxin from gram-negative bacteria (29).

Chilled-water spray humidification systems are used in textile-producing facilities to provide humidity and temperature control. To humidify air, ambient outside air is filtered and drawn through a chilled-water (16°C) spray. After humidification, large water droplets are removed by passage through aluminum demister vanes set at angles to the direction of the air flow.

Several years ago employees in a portion of a facility using chilled-water spray humidification had complaints similar to hypersensitivity pneumonitis or humidifier fever (3, 11, 14–17, 25, 29, 31) or both. Preliminary studies by Reed et al. showed that a putative agent of disease was present in bicarbonate extracts of a brown "slimelike" biomass growing on the demister vanes in the humidification system (28, 34).

This study was undertaken to isolate and characterize the serologically reactive material present in bicarbonate extracts of the biomass. The isolation and characterization of a biologically active endotoxin are described.

MATERIALS AND METHODS

Raw biomass. The raw biomass was a brown gelatinous sheath which grew to a depth of 1.0 mm on the aluminum demister vanes of the air washers. The biomass was recovered by mechanical scraping of the demister vanes.

Bicarbonate extract. The bicarbonate extract was prepared by the method described by Reed et al. (28, 34). The biomass was centrifuged at 400 × *g* at room temperature for 10 min. After the supernatant fluid was discarded, the precipitate was resuspended in 0.75 M sodium bicarbonate buffer, pH 8.6, using a ratio of 10 ml of buffer to 1.0 g (wet weight) of precipitate. The mixture was stirred for 24 h at room temperature and centrifuged at 800 × *g* for 10 min at room temperature, and the supernatant fluid was recovered. The supernatant fluid was dialyzed against deionized water at room temperature for 24 to 48 h, using a minimum of 20 changes of deionized water. By atomic absorption spectroscopy analyses, the concentration of sodium in the dialysate was below 5 ppm (5 μg/ml). The dialysate was then filtered through a 0.45-μm filter (Millipore Corp., Bedford, Mass.) by high-pressure filtration, using Spectra stirred cells (Fisher Scientific Co., Chicago, Ill.). The material passing through the 0.45-μm filter was lyophilized.

LPS extraction. The lipopolysaccharide (LPS) fractions were recovered by using a 5-min phenol-water extraction as described by Westphal and Jann (37). The water layer was recovered and dialyzed against deionized water for 24 to 48 h at room temperature, using 30 changes of water. After dialysis, the concentration of phenol in the dialysate was determined by UV adsorption at 278 and 283 nm, using a phenol-water calibration curve. The dialysates contained ≤2.0 ppm (≤2.0 μg/ml) of phenol. By high-pressure filtration, the LPS fractions were filtered through a 0.45-μm filter (Millipore). The eluate was then concentrated 10-fold by high-pressure filtration, using Spectra stirred cells with 10,000-molecular-weight cutoff filters. The retained LPS fractions with molecular weights of >10,000 were lyophilized.

Carbohydrate analyses. The total carbohydrate concentration in the extracts was determined by the phenol-H_2SO_4 method (13). Total hexose sugars were ascertained by the anthrone reaction (29). Pentose and methyl pentose sugars were determined by the cysteine-H_2SO_4 method of Dische (12).

To identify individual sugars present in extracts, samples were also analyzed by gas-liquid chromatography, using alditol acetate derivatives. One to 4 mg of sample was blanketed with nitrogen and hydrolyzed for 48 h in 2.0 ml of 2.0 N HCl at 100.5°C, using Teflon-lined digestion tubes

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(Supelco, Bellefonte, Pa.). After cooling to room temperature, the digested samples were extracted three times with 2.0 ml of UV-grade hexane. The extracted samples were dried by using nitrogen at 75°C and vacuum desiccation for 2 h at room temperature. A 1-ml amount of 1.0 M NH_4OH with 4.0 mg of sodium borohydride per ml was added to each sample, and the samples were allowed to react for 40 min at room temperature. After neutralization with glacial acetic acid, the samples were reduced to a viscous liquid with nitrogen at 75°C. A 2-ml portion of methanol-benzene (4:1, vol/vol) was added, and the samples were heated at 90°C for 5 min. After cooling, samples were again evaporated with nitrogen at 37°C. Five additional evaporations with 2.0 ml of methanol at 37°C removed the boron as methyl borate. After the samples were dried in a vacuum desiccator at room temperature, acetic anhydride (1.5 ml) was added and the mixtures were heated at 100°C for 30 min. After they were dried with nitrogen at 37°C, samples were extracted with chloroform-water (1:1, vol/vol).

All chloroform layers were extracted five times with 1.0 ml of water and evaporated to dryness, using nitrogen at 37°C. Dried samples were diluted to 100 μl with chloroform and analyzed on a Varian 2100 (Varian Instruments, Palo Alto, Calif.), using a flame ionization detector. Samples were chromatographed on a glass column (6.0 ft [ca. 1.8 m] by 2.0 mm [inside diameter]) packed with SP-2330 on 100/120 Suplecoport packing (Supelco). The flow rate was 30 ml/min, with sensitivity of $10^{-10} \times 4$ attenuation. To determine percentage of hydrolysis and derivatization, known solutions of 10 neutral sugars and inositol (internal standard) were assayed in parallel with the test samples. To validate retention times, commercially available alditol acetate-sugar mixtures (Supelco) were used in the assay.

Hexosamine analyses. Hexosamines were identified and quantitated, using the gas-liquid chromatography methods described above. The length of the chromatographic column was shortened to 3.0 ft (ca. 91 cm) for hexosamines. The method of Aminoff et al. (1) was also used to determine *N*-acetylhexosamines.

Nucleic acid analyses. The concentration of nucleic acids in the extracts was determined by the diphenylamine reaction described by Burton (9) and by UV spectroscopy (20). To identify nucleic acid bases, reverse-phase high-performance liquid chromatography was used. Extracts were chromatographed on a Bondapack μC18 column (Waters Associates, Milford, Mass.), using a linear gradient from 0.02 M KH_2PO_4, pH 5.7, to 100% methanol. A Perkin-Elmer LC55 UV detector at 254 nm was used for detection (2).

Phosphorus analyses. The concentration of phosphorus was determined by the ammonium molybdate method of Snell and Snell (33) and X-ray fluorescence spectroscopy (21).

Lipid analyses. Samples of the extracts were placed in Teflon cups and reconstituted in 5.0 ml of 5% NaOH in 50% aqueous methanol. By using stainless-steel acid digestion bombs, the mixtures were saponified for 60 min at 100°C. After cooling, the pH of the saponified material was lowered to 2.0 with 6.0 N HCl. The free fatty acids were recovered by sequential extraction with 10 ml of chloroform-hexane (1:4).

The solvent layer containing the free fatty acids was combined and evaporated to dryness, using nitrogen at 37°C. Methyl esters of free fatty acids were formed by adding 5.0 ml of 10% (vol/vol) boron trifluoride and heating the mixture for 10 min at 80°C. The methyl esters were analyzed by gas-liquid chromatography, using a glass column (12 ft [ca. 3.7 m] by 2.0 mm [inside diameter]) of 3% methyl silicone (SP-2100 DOH; Supelco) on 100/120 Supelcoport supports. To determine qualitative retention times, a reference standard consisting of 22 methyl esters of bacterial fatty acids (Bacterial Acids M.C.; Supelco) was used. In quantitative analyses, seven even-numbered saturated and unsaturated fatty acids were carried through the hydrolysis and esterification protocol in parallel with the test samples.

Protein analyses. The presence of protein in the extracts was ascertained by two methods. In one method, the presence of amide-carboxyl linkages was determined by UV absorption between 270 and 285 nm (20). In the second method, protein was determined by the Bio-Rad (Bio-Rad Laboratories, Richmond, Calif.) protein assay (6, 10).

Trace metal analyses. The possible presence of trace metal contaminants in the extracts was determined by atomic absorption spectroscopy.

Moisture analysis. The moisture content of lyophilized extracts was determined by standard methods in a coulorimetric moisture analyzer (Dupont Instruments, Seabrook, Del.).

Fractionation of LPS antigen. The LPS extract was fractionated by anion-exchange chromatography using a DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) column (10 by 1.5 cm). All extracts were equilibrated with the starting buffer, 0.05 M potassium phosphate buffer (pH 7.2) with 0.15 M NaCl, and eluted with a stepwise salt gradient (0.15 to 1.0 M NaCl in the phosphate buffer).

***Limulus* assays.** By using a standard reference endotoxin from *Escherichia coli* O127:B8, the in vitro endotoxin potential of the extracts was determined by the standard *Limulus* amoebocyte lysate assay (23). Commercially available Pyrostat kits (Worthington Diagnostics, Freehold, N.J.) were used in the study. The percent endotoxin (wt/wt) was computed by dividing the nanograms of endotoxin equivalent per milliliter by the nanograms of extract per milliliter tested and multiplying by 100.

Rabbit pyrogenicity tests. White New Zealand rabbits weighing 2.0 to 2.5 kg were injected via the marginal ear vein, using an approved Food and Drug Administration protocol (34). The change in body temperature was observed over a 2- to 4-h period. The minimal pyrogenic dose was that which induced a rise in temperature of ≥0.6°C in all test rabbits.

Pooled precipitin-positive sera. Serum samples from sub-

TABLE 1. Colorimetric chemical analyses of serologically reactive extracts

Extract	% (wt/wt)										
	Total carbohydrate	Hexose sugars	Pentose sugars	Methyl pentose sugars	Hexosamines	Phosphorus (PO_4)	Protein	Nucleic acids	Water	Iron	Chromium
Bicarbonate	26.5	15.7	1.7	2.6	2.4	1.62	18.9	10.0	12.0	1.0	1.5
Bicarbonate and phenol	40.7	31.0	2.7	2.8	3.2	1.62	0	10.0	13.0	0	0
Phenol extract of raw biomass	73.4	54.3	4.4	7.4	4.3	1.50	0	9.0	14.0	0	0

TABLE 2. Lipid analyses of the serologically reactive extracts

Extract	% of total fatty acids											
	Methyl undeconate 11:0	Methyl laurate 12:0	Methyl tri-deconate 13:0	Methyl myristate 14:0	Methyl penta-deconate 15:0	Methyl palmitate 16:0	Methyl palmit-oleate 16:1	Methyl hepta-deconate 17:0	Methyl stearate 18:0	Methyl oleate 18:1	Methyl nona-deconate 19:0	Methyl arachidate 20:0
Bicarbonate	Tr	4.0	Tr	6.3	Tr	41.0	8.0	Tr	13.2	20.0	Tr	3.4
Bicarbonate and phenol	Tr	4.2	Tr	7.9	Tr	30.3	11.6	Tr	18.6	22.4	Tr	2.3
Phenol extract of raw biomass	Tr	2.9	Tr	12.8	5.3	24.8	3.3	Tr	23.6	24.0	Tr	Tr

[a] Tr = ≤2.0% of total fatty acids.

jects with proven lung disease (n = 3) and subjects strongly suspected of having lung disease (n = 3) were pooled for use in serological studies.

CIEP. Counterimmunoelectrophoresis (CIEP) as described by Bartram et al. (4) was used to determine the presence or frequency or both of serological reactivity directed toward organic material present in extracts.

mELISA. Standard indirect Micro-enzyme-linked immunospecific assays (mELISA) were used to confirm other serological tests or demonstrate enrichment of serologically reactive material (7, 36). Diluted (1:1,000) precipitin-positive sera were used as the primary antibody. Alkaline phosphatase-conjugated anti-human immunoglobulin (Sigma Chemical Co., St. Louis, Mo.) was used in the amplification step.

RESULTS

Previous studies demonstrated that serologically reactive material was present in bicarbonate extracts of the demister vane biomass. Hence, studies were undertaken to ascertain the constituents of the extract by using conventional wet-chemical methods. Approximately 10% of the total biomass could be extracted into the bicarbonate buffer. The bicarbonate extract contained carbohydrates, hexosamines, phosphorus, protein, and nucleic acids (Table 1). Even-numbered saturated and unsaturated fatty acids were also demonstrated by gas-liquid chromatography (Table 2). Atomic absorption spectroscopy showed the presence of iron and chromium. By using Sephacryl S-300 columns, it was shown that the extract contained high-molecular-weight, metal-containing complexes (data not shown).

To avoid the possibility that the metal contaminants would influence sensitive analytical techniques, attempts were made to prepare an extract free of metal contamination. Because the bicarbonate extract contained constituents similar to a bacterial LPS, classical phenol-water extraction was used to fractionate the bicarbonate extract.

To optimize the phenol extraction procedure, samples of the bicarbonate extract were further extracted for periods of 1 to 45 min. After lyophilization, each extract was weighed to determine the percent recovery and tested for serological reactivity by CIEP, using pooled precipitin-positive sera. If the samples were extracted for longer than 5 min, the

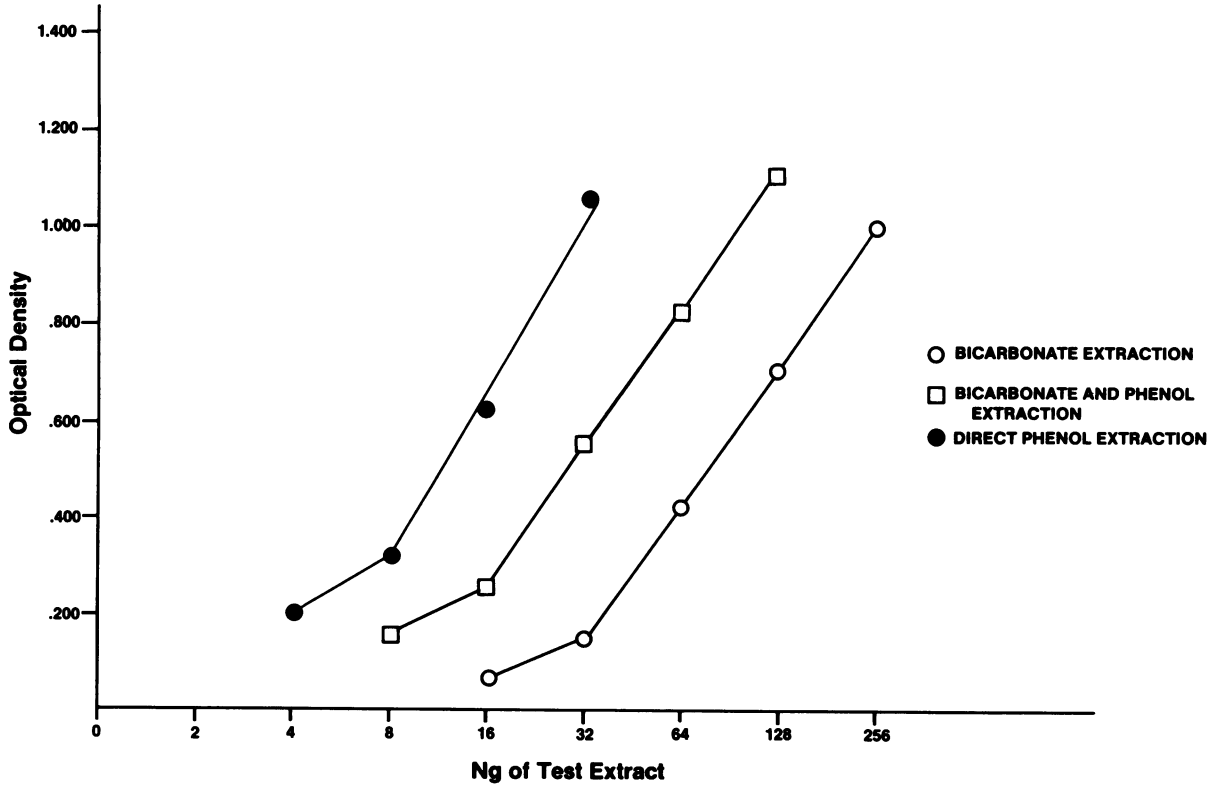

FIG. 1. Concentration of antigen per unit weight in various extracts when assayed in mELISA, using a standard dilution of precipitin-positive sera.

serological reactivity was lost and the percent recovery was reduced.

With the 5-min phenol extraction, 39% (wt/wt) of the bicarbonate extract could be recovered in the water layer. The constituents of the bicarbonate-plus-phenol extract were similar to those of the bicarbonate extract, but lacked the protein fraction. Atomic absorbance spectroscopy showed that the extract was free of chromium and iron.

Because of the enrichment observed when the bicarbonate extract was extracted into phenol, it was assumed that direct phenol extraction of the raw biomass would further increase the yield of serologically reactive material. With direct phenol extraction, 10 to 14% (wt/wt) of the biomass was recovered in the water layer. An increase in carbohydrates and lipids was also observed (Tables 1 and 2).

Studies were conducted to determine the efficiency of extraction in terms of biological activity and serological reactivity. The *Limulus* assay was used to assess the endotoxin potential of each extract. mELISA were used to determine the amount of serological reactivity per unit weight of extract.

In the *Limulus* assay, the bicarbonate extract had an endotoxin equivalent of 0.496 ng/ml or 0.396% (wt/wt) endotoxin. Further extraction of the bicarbonate extract with phenol resulted in an enhancement of endotoxin activity to 1.06 ng/ml or 0.848% (wt/wt) endotoxin. Direct phenol extraction of the biomass yielded an endotoxin equivalent of 2.76 ng/ml or 2.2% (wt/wt) endotoxin.

Additional studies were conducted to determine whether the serological reactivity was influenced by the method of extraction. The data show that the serological activity was low in the bicarbonate extract. Significant enhancement of reactivity was observed when the bicarbonate extract was extracted into phenol. The greatest serological reactivity was observed in the phenol extract of the raw biomass. A comparison of the slopes of the three lines shows that they are similar if not identical (Fig. 1).

Since direct phenol extraction of the biomass yielded an extract with the highest endotoxin activity and serological reactivity per unit weight, it was assumed that this preparation was the best source of material to be used in confirmatory biological and analytical studies.

With the phenol extract of the biomass, additional analytical methods were used in the confirmatory studies. Alditol acetate derivitization of the LPS hydrolysates yielded nine sugars, with galactose and glucose present in high concentrations (Table 3). Lipid studies showed the presence of even-numbered saturated and unsaturated fatty acids. In addition, hydroxylated (3-OH14:0) and branched-chain (i 15:0, i 17:0, a 15:0, and a 17:0) fatty acids were observed in the extract (Table 4).

The nucleic acids present in the phenol extract of the biomass were found to be RNAs. Microgram levels of cytosine, uracil, guanine, and adenine were demonstrated by high-performance liquid chromatography. Thymidine was present in only trace concentrations. Based on PO_4 concentration and base pair composition, the calculated RNA concentration was 9.0%.

After confirmation of the chemical constituents present in the phenol extract of the biomass, the endotoxin potential of the extract was confirmed by in vivo rabbit pyrogenicity assays. Rabbits (n = 3) were inoculated over a wide concentration range (0 to 5,000 ng/kg). One of three rabbits responded when inoculated with 1,250 ng of extract per kg. All three rabbits in another test group demonstrated a positive pyrogenic response at 2,500 ng/kg.

TABLE 3. Carbohydrate constituents of the raw biomass extract and column-purified fractions

Extract/fraction	% (wt/wt)																
	Glyceraldehyde	Rhamnose	Fucose	Ribose	Arabinose	2-Deoxyribose	Mannose	Galactose	Xylose	Glucose	Heptose	*N*-Acetylgalactosamine	*N*-Acetylglucosamine	Phosphorus (PO_4)	RNAs	Water
Phenol extract of raw biomass	—[a]	11.6	2.4	1.8	2.2	0.2	15.6	12.2	0.6	18.4	3.3	10.0	2.0	2.73	9.0	14.0
Fraction 1	1.3	3.7	1.0	—	0.1	0.6	6.4	7.9	0.2	15.4	2.7	16.7	9.0	1.22	<0.15	20.0
Fraction 2	0.4	2.9	0.5	0.5	—	2.2	4.9	6.6	0.2	10.6	2.6	10.3	4.3	3.87	3.3	26.0
Fraction 3	ND[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	10.55	47.8	30.0

[a] —, Not detected.
[b] ND, Not done.

TABLE 4. Lipid constituents of the raw biomass extract and column-purified fractions[a]

Extract/fraction	% (wt/wt) of total fatty acids																				
	11:0	12:0	13:0	13:1	14:0	2OH 14:0	3OH 14:0	14:1	15:0	i 15:0	a 15:0	15:1	16:0	16:1	17:0	i 17:0	a 17:0	17:1	18:0	18:1	19:0	19:1	20:0
Phenol extract of raw biomass	T[b]	2.9	Tr	—[c]	12.8	—	Tr	—	5.3	Tr	Tr	—	24.8	3.3	Tr	Tr	Tr	—	23.4	24.0	Tr	—	Tr
Fraction 1	—	7.8	—	—	5.3	—	Tr	—	2.6	Tr	Tr	—	34.6	7.1	Tr	Tr	Tr	—	14.9	14.9	Tr	—	—
Fraction 2	—	5.4	—	—	4.8	—	Tr	—	Tr	Tr	Tr	—	32.2	9.5	Tr	Tr	Tr	—	17.9	20.3	—	—	Tr

[a] Analysis was not done for fraction 3.
[b] Tr = ≤2.0% of total fatty acids.
[c] —, Not detected.

To ensure that the endotoxin activity and the serological reactivity were associated with the LPS fraction of the phenol extract of the biomass, the extract was fractionated by anion-exchange chromatography (Fig. 2). Three fractions were recovered. Fraction 1 contained the LPS and phosphorus but lacked nucleic acid base pairs (Tables 3 and 4). The second fraction also contained LPS but was contaminated with 3.3% RNA. Because this fraction contained RNA, it was not tested further. The last fraction (fraction 3) contained 47.8% RNA.

Fractions 1 and 3 were tested for serological reactivity and endotoxin potential. When tested by CIEP with pooled precipitin-positive sera, serological reactivity was demonstrated only in fraction 1. Fraction 1 had a computed endotoxin equivalent of 2.0 ng/ml, or 2.6% (wt/wt) endotoxin. Fraction 3 had only 0.5% (wt/wt) endotoxin.

To demonstrate a putative association between the endotoxin and disease, serum samples were tested for serological reactivity directed toward column-purified endotoxin by CIEP. All subjects with proven disease and a high proportion of the suspect group had antibodies directed toward the endotoxin (Table 5). A smaller number of exposed, precipitin-positive subjects had antibodies directed toward the endotoxin. All serological reactions were confirmed by mELISA, using alkaline phosphatase-labeled anti-human immunoglobulin.

DISCUSSION

The presence of chromium and iron in the bicarbonate extracts was not unexpected. Sodium dichromate is routinely used as a rust inhibitor in chilled-water spray systems. Iron is present in both the chilled water and rust in the system. Bacterial LPS are negatively charged macromolecules and are routinely isolated in salt form complexed with metal cations (19). In this case, the chromium and iron may be expected to initiate the formation of LPS-cation complexes.

Phenol extraction has been used by several investigators to extract LPS and nucleic acids from intact bacteria (27, 37). The duration of contact with phenol necessary to extract serologically reactive LPS varies with bacterial species (27). The 5-min phenol extraction used in this study was similar to that used to extract LPS from *Salmonella typhi* O901 (27).

It is unlikely that phenol extraction alters the serological reactivity of the antigen. Comparison of the serological reactivity of the three extracts showed that there was an increaase in the amount of antigen per unit weight. Moreover, the slopes of the three lines were identical. If different antigens had been extracted by phenol, the slope of the line would have changed (38).

The presence of RNA in the phenol extract was not unexpected since the technique is often used to partition nucleic acids and protein between phenol and water (37). The concentration of RNA observed in this study (9.0%) was lower than that found (40 to 60%) when intact bacteria are used in phenol extraction (37). There was some discrepancy in the amount of PO_4 present in the extracts. This may be related to the fact that ammonium molybdate assays were replaced by X-ray fluorescence assays, a more sensitive method, later in the study.

The relationship between the RNA and the endotoxin is unclear. It is unlikely that the molecules were complexed since both endotoxins and RNA are highly anionic (27). It is clear, however, that the purified RNA fraction (fraction 3) lacked serological reactivity and endotoxin potential. The latter observation fails to confirm the report of Elin and

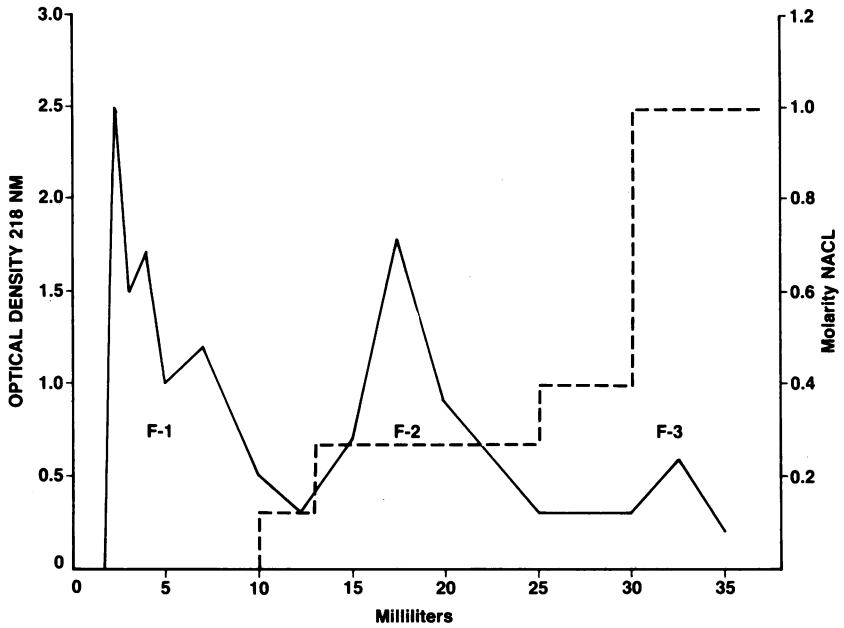

FIG. 2. Chromatographic profile of the phenol extract of the raw biomass, using anion-exchange column chromatography and stepwise salt gradients (Dashed line).

Wolff, who suggested that polynucleotides could activate the *Limulus* assay (16).

The chemical constituents of the column-purified biomass endotoxin were reported to be present in the O-polysaccharide, R-core, and lipid A moiety of other bacterial endotoxins (25). In the absence of nucleic acids, the concentration of phosphorus reported in this study is similar to concentrations reported in lipid A fractions (8).

There may be some minor qualitative and quantitative problems associated with alditol acetate derivitization used to identify individual sugar residues. Although most sugars could be identified with a reasonable degree of certainty, the designation of a heptose should be considered tentative. It is conceivable that the molecule could be a heptulose or a dideoxyhexosamine. All three molecules were reported to be present in some bacterial endotoxins (22, 26). Moreover, the reported concentrations of select sugars may be slightly lower than expected. During the hydrolysis and derivitization, ribose and 2-deoxyribose may be degraded to glyceraldehyde, thus reducing the reported concentrations of these sugars.

TABLE 5. Serological reactivity directed toward the bicarbonate extract and the column-purified endotoxin, using CIEP

Study group	No. tested	Precipitating antibodies in bicarbonate extract	Serological reactivity to purified endotoxin			
			No. positive	% Positive	No. negative	% Negative
Hypersensitivity pneumonitis[a]	7	+	7	100	0	0
Suspect[b]	20	+	19	95.0	1	5.0
Normal[c]	73	+	57	78.0	16	28.0
Normal	50	−	1	2.0	49	98.0
Non-employees	50	−	1	2.0	49	98.0

[a] Biopsy diagnosis.
[b] Clinical symptoms but no pulmonary function or radiological abnormalities.
[c] Employees with no clinical history and no pulmonary or radiological abnormalities.

The biological activity of the column-purified endotoxin was lower than that reported for *Salmonella* spp. or *E. coli*. When compared with an *E. coli* standard curve in the *Limulus* assay, the isolated endotoxin was only 2.2% as active on a weight basis. In the rabbit pyrogenicity assays, the concentration necessary to elicit the minimal pyrogenic response was 2,500 ng/kg. A similar pyrogenic response can be elicited with 10^{-3} to 10^{-6} μg of *Salmonella* endotoxin per kg (27). Despite the low biological activity, the endotoxin is considered to be a pyrogen by Food and Drug Administration standards (35).

The isolation of a biologically active endotoxin from the biomass was not unexpected. Generally, gram-negative bacteria have low nutritional requirements and can exist even in minimal salt solutions (5). Hence, the biomass may provide a suitable environment for the growth of gram-negative bacteria.

It is clear that all subjects with proven disease and a high proportion of the suspect group had antibodies directed toward the endotoxin. The presence of antibodies in a normal, exposed working population was not unusual. Previous studies demonstrated that only a fraction of an exposed population ever develop disease (3, 29). The frequency of serological reactions (78%) in the normal exposed group was higher than previously reported for outbreaks of hypersensitivity pneumonitis but similar to precipitin frequencies observed in humidifier fever (24, 32).

It is unlikely that the serological reactions were due to nonantibody-LPS interactions. CIEP has been used to circumvent such problems (4). In addition, alkaline phosphatase-labeled anti-human immunoglobulin was used in m-ELISA to confirm the nature of the serological reactions.

Although the data strongly suggest that the endotoxin is the putative agent of lung disease, the role of the endotoxin in the disease process cannot be discerned from this report. However, a serologically reactive, endotoxin-containing *Cytophaga* species has been isolated from the biomass (C. A.

Liebert, F. H, Deck, M. A. Hood, K. Bishop, F. L. Singleton, and D. K. Flaherty, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C36, p. 317). The structure of the serologically reactive *Cytophaga* endotoxin is similar to that described for the biomass endotoxin (18). Further, human inhalation challenge with either the purified biomass or *Cytophaga* endotoxin evoked clinical and respiratory symptoms similar to those observed in affected workers (18).

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